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Original Article

The role of autophagy in lung ischemia/reperfusion injury after lung transplantation in rats

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Abstract: Background: The aim of this study was to explore the role of autophagy in the cold I/R injury following lung transplantation. Methods: The rat orthotopic lung transplantation model was established to perform the level of autophagy in the cold I/R injury in this study. The pretreatment of inhibitor (3-Methyladenine [3-MA]) and activator (rapamycin [RAPA])of autophagy were performed to assess the role of autophagy in the cold I/R injury following lung transplantation in rats. Results: After lung transplantation, the autophagy, lung cell apoptosis and lung injury were aggravated and peaked at 6 h following the transplantation. The inhibition of autophagy by 3-MA induced downregulated of autophagy, decreased cell apoptosis. Meanwhile, the lung injury, which was indicated by calculating the peak inspiratory pressure (PIP), pulmonary vein blood gas analysis (PO_2) and ratio of wet to dry in lung (W/D), was ameliorated after treatment with 3-MA. The activation of autophagy by RAPA causing the upregulated of autophagy and apoptosis of lung cells, and enhanced the lung injury. Conclusion: All the results suggested that the autophagy was involved in the cold I/R injury in lung transplantation model, and played a potential role on the regulation of I/R injury after lung transplantation.

Keywords: Autophagy, lung ischemia/reperfusion injury, lung transplantation

Introduction

Lung transplantation is the major therapeutic strategy for advanced stage lung diseases. However, the statistics show that the 1-year survival rate after lung transplantation is less than 80%, which mainly due to the primary graft dysfunction (PGD) [1]. Ischemia/reperfusion (I/R) injury is described as the major cause of PGD, which has received a great deal of attentions on its function in the treatment of lung diseases [2, 3]. I/R injury is the phenomenon of cellular damage in the organ, which is caused during the hypoxia or anoxia becomes exacerbated when the oxygen and pH are restored [4]. It is genuarry considered that the I/R injury is a process leading to the deaths in parenchymal cells and progresses to the inflammatory response that involved in the cytotoxic mechanisms [5]. The lung I/R injury usually occurs in different kinds of clinical conditions, including lung transplantation, pulmonary throm-boendarterectomy, heart surgery, cardiopulmonary bypass, post-resuscitation for circulatory arrest, hemorrhagic shock and so on [6, 7]. It has been reported to cause the elevated mortality of lung diseases, especially the mortality following lung transplantation [8].

Autophagy is an intracellular self-digesting pathway to remove the abnormal malformed proteins, organelles and unnecessary cellular contents with the lysosomal digestion [9]. Accumulated evidences demonstrated that the autophagy plays a crucial role in some pathophysiological status, such as cancer, immunity, infection and many neurodegenerative diseases [10]. Studies suggested that the autophagy is a protective process in cells, but it can also cause deaths of cells [11, 12]. Wei *et al.* suggested that the autophagy takes place during the cell death in the nervous system and some neurodegeneration cases [13]. Carol and his colleagues demonstrated that the autophagy can maintain the survival of myocardial cells under the stressful condition, but it also can

Table 1. The details of grouping design

Group	Perfusate	Cold ischemia preservation time (h)	Reperfusion time (h)	Sample number
EXP2	LPD	6	2	6
EXP6	LPD	6	6	6
EXP12	LPD	6	12	6
CON1	LPD	0	-	6
CON2	LPD	6	-	6
3-MA	3-MA+LPD	6	6	6
RAPA	RAPA+LPD	6	6	6
CON	LPD	6	6	6

drive the cells to die if the cellular stress is unmanageable [14]. In the previous studies, autophagy has been reported to involve in the lung warm I/R injury process [15]. However, the role of autophagy in the lung cold I/R injury following lung transplantation has never been investigated.

In the current study, we aimed at to set up the rat lung transplantation model to perform the clinical signification of autophagy in the lung cold I/R injury in this model.

Materials and methods

Animals

Total of 120 specific pathogen-free Sprague-Dawley male rats (weight 300±25 g) were purchased from the Animal science laboratory of Nanchang University. The animal certification number was JZDWNO (2012, 0498). All these animals were housed and fed with the conventional animal facilities under the controlled environment, and kept under a 12/12 h light/dark cycle condition. Total of the animals were obtained the care in accordance with the Principles of Animal Use Committee (China).

Reagents and antibodies

The low-postassium dextran (LPD) was purchased from SeeBio. The 3-Methyladenine (3-MA) and rapamycin (RAPA) were obtained from Selleckchem. In the present study, a rabbit polyclonal antibody against light chain (LC) 3 was purchased from Sigma, which was used in the fluorescent staining. The antibodies, including anti-LC3, anti-Beclin-1 and anti-β-actin used in the western blotting were purchased from Cell Signaling Technology.

Establishment of rat orthotopic lung transplantation model

All the rats were randomly divided into donor group and receptor group. The rats of donor group were anesthetized by intraperitoneal injection with 1 ml volum of 10% chloral hydrate, then processed with the orotracheal intubation and connected to the rodent ventilator with the optimal parameters (frequency of 75-80 times per min, total volume of 10 mL/kg and inspiration time/exoiratory time of 1:2.0). An thoracic and abdominal operation was carried out, and 1 mL of heparin (1250 IU/kg) was injected into the postcava for systemic antocoagulation. The LPD solution was injected into the pulmonary trunk by catheter. The blood letting was performed in the lung tissue by cutting the auricle and postcava. The combined heart and lung tissue was collected after finished the blood letting. Then the left donor lung was isolated from these combined tissue, implantation of the cuffs and stored into the LPD solution at 4°C for 6 h. The orthotopic lung transplantation in the rats of receptor group was performed by using the cuffs in the pulmonary artery, pulmonary vein and bronchia. After the operation, all the rats were carefully sutured, and the indices of lung function were investigated at the time designed in this study.

Grouping design

Total of the rats in the present study were randomly divided into each of the groups. In order to examine the influence of different reperfusion time (2 h, 6 h and 12 h) on the autophagy following the lung transplantation, three experimental groups were designed: EXP2, EXP6 and EXP12. Two control groups was prepared: CON1 and CON2. To explore the role of autophagy in I/R injury after lung transplantation, the regulations of autophagy by autophagic inhibitor (3-MA) and activator (RAPA) were performed in this study. Two experimental groups were prepared: 3-MA group and RAPA group. The control group (CON group) was established without agent. The details of the grouping design were listed in **Table 1**.

Western blotting

The expression of Beclin-1 and LC3 were examined by western blotting to indicate the level of

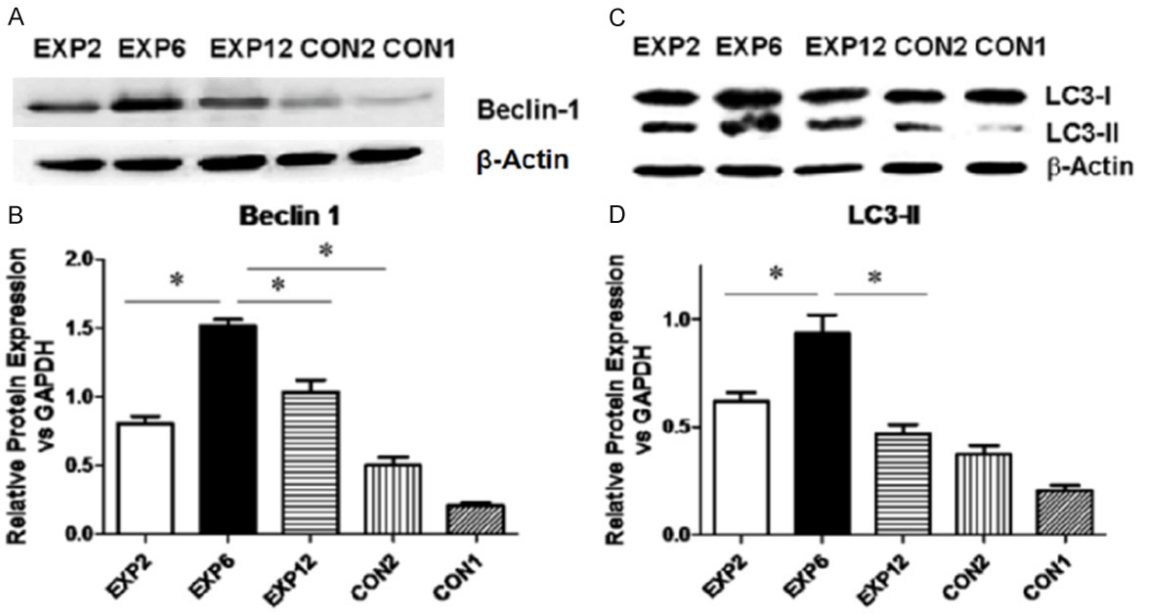

Figure 1. The expression of Beclin-1 and LC3 measured by western blotting in rats lung transplantation model. A. Representative immunoblots of Beclin-1 in lung. B. Beclin-1/GAPDH ratios were quantitated by densitometry. C. Representative immunoblots of LC3 in lung. D. LC3-II protein levels were quantitaed by normalizing to GAPDH.

autophagy in rat lung transplantation model. Total of the proteins were extracted with SDS, then separated with SDS-PAGE. The proteins were all electrotransferred to the polyvinylidene fluoride (PVDF) membranes. The incubation of membranes with antibodies were performed for 1-2 h. The bands of protein were matured by using the chemiluminescence (ECL) reagents. Moreover, the β-actin protein was applied to act as the control protein.

Immunofluorescence

In order to locate the autophagosomes and lysosomes in lung cells, we applied immunofluorescence in the present study. The lung tissue samples was fixed with the 4% paraformaldehyde, then washed with PBS buffer (pH=7.4). Lung sections were sliced after the paraffin embedding. Sections used in the analysis were selected randomly. All these sections were incubated with the rabbit monoclonal antibody against Beclin-1 (B6186 1:200) and polyclonal antibody against LC3 (L8918 1:300) at 4°C overnight. After washed with PBS buffer, the sections were incubated with the second antibodies at room temperature for 1 h in a lucifugal environmental. Then the sections were counterstained and rinsed, then observed with the OLYMPUS Fluoview flv1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Three representative fields were collected for scanning and quantification under the high magnification from each of the lung tissue sections. The images were obtained by using the Image-pro plus 6.0 software, and the quantitative analysis was carried out by measuring the value of optical density (OD).

The apoptosis of lung cells

In the current study, TUNEL method was used to perform the apoptosis of lung cells following lung transplantation. The TUNEL staining positive sections were isolated from each groups, and the positive cells were accounted by using microscope. The ratio of positive cells in total 500 cells represents the apoptotic index (AI).

The indices of lung function

We recorded the indices of lung function, including peak inspiratory pressure (PIP), pulmonary vein blood gas analysis (PO_2) and ratio of wet to dry in lung (W/D). The PIP was measure by using the respiratory pressure meter extech 40749 (Extech, USA). To analyze the PO_2 in lung vein, blood gas analyzer (IRMASL, USA) was applied in this study. The W/D ratio was investigated with 0.5 g lung tissue by using the constant temperature oven.

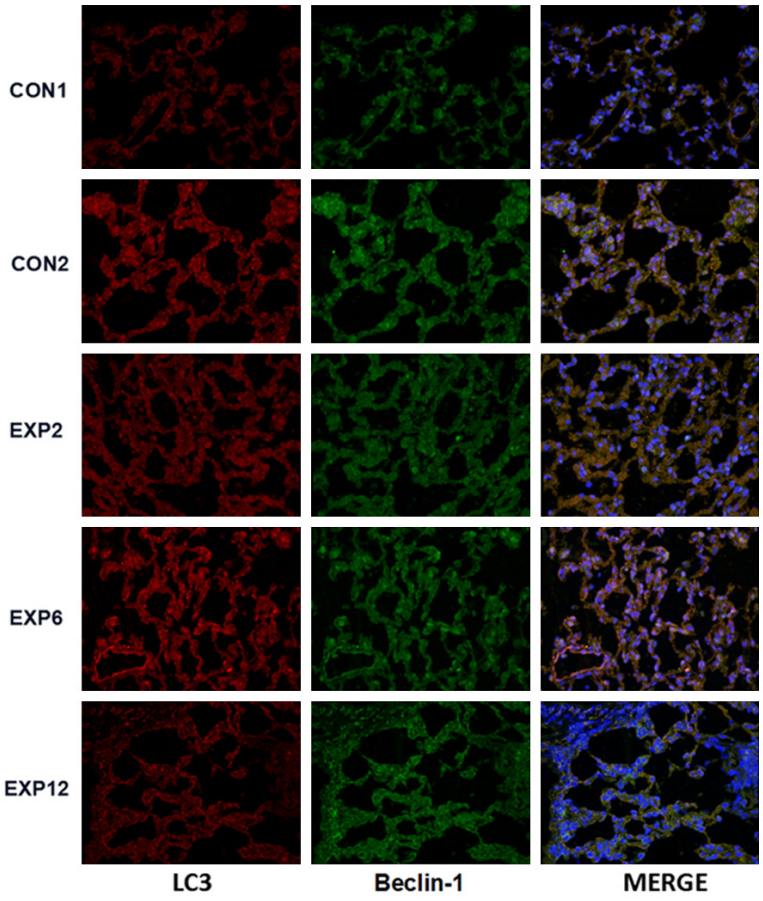

Figure 2. The colocalization of autophagosome and lysosome in lung samples. The representative image of double immunolabeling against LC3 (red) and Beclin-1 (green) confocal microscopy suggested the fusion of autophagosomes with lysosomes.

Statistical analysis

All the data were expressed by the means of ± SD. The statistical analyses were performed by using the SPSS 18.0 software. The comparison between different groups was analyzed by one-way ANOVA analysis. Only the results with *P* value of <0.05 were considered statistically significant.

Results

The effect of I/R injury on autophagy, lung cell apoptosis and lung function following lung transplantation

To investigate the autophagy level in lung tissue after transplantation, the expression of Beclin-1, LC3 protiens were measured by western blotting. From the results of western blotting in **Figure 1**, we found that the Beclin-1 and LC-II expression were upregulated and were significantly higher in EXP6 group than that in the other groups after lung transplantation ($P<0.05$). The location of autophagosomes and lysosomes in lung cells were investigated to confirm the autophagy level in lung tissue, the results of immunofluorescence revealed that the autophagic flux was increased following lung transplantation, and was peaked at 6 h after reperfusion (**Figure 2**).

The apoptosis of lung cells following lung transplantation was investigated by using the TUNEL assay. The results shown that the cell apoptosis was increased after transplantation with the growth time. The highest apoptosis was performed after 6 h following lung transplantation in EXP6 group. The differences of cell apoptosis between EXR6 group with EXP2 and EXP12 group were statistically significant ($P<0.05$, **Figure 3**).

The lung function was performed by calculating the major indices, including PIP, PO_2 and W/D ratio. All the value of these indices were listed in **Table 2**, and the differences were calculated with SPSS software (**Figure 4**). All the value of PIP and W/D ratio in the experimental groups were higher than that in the control groups, which suggested the serious lung injury in the experimental groups. The PIP and W/D ratio were higher in EXP6 group than that in the other experimental groups ($P<0.05$). While the PO2 was lower in EXP6 group compard with the EXP2 and EXP12 groups ($P<0.05$). All these data suggested that the injury of lung function was increased after lung transplantation, and was most serious at the 6 h after the operation, then declined thereafter.

The effects of agents on I/R injury by regulating autophagy in lung tissue

The autophagy was regulated with the agents of 3-MA and RAPA. 3-MA and RAPA were the

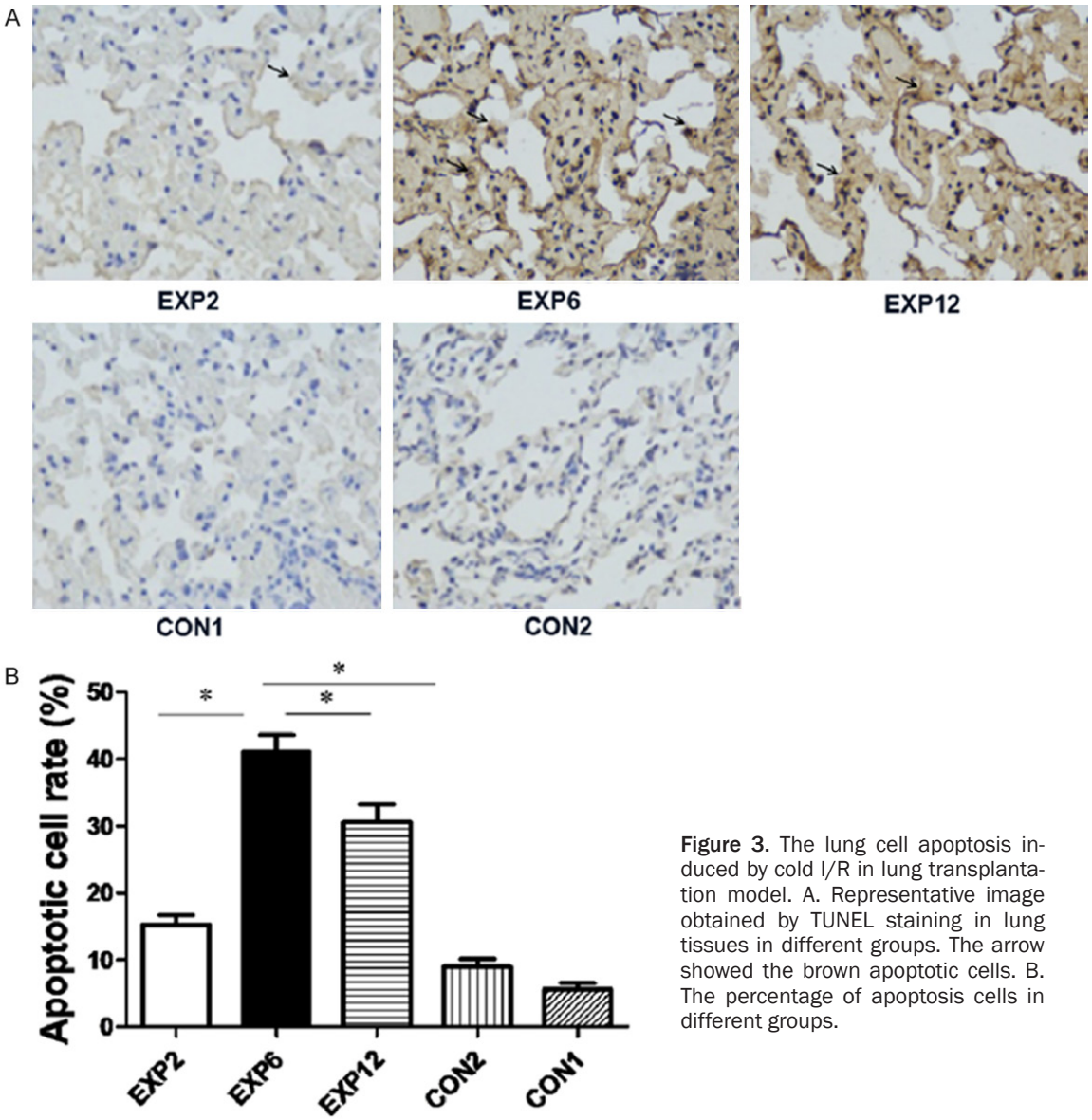

Figure 3. The lung cell apoptosis induced by cold I/R in lung transplantation model. A. Representative image obtained by TUNEL staining in lung tissues in different groups. The arrow showed the brown apoptotic cells. B. The percentage of apoptosis cells in different groups.

Table 2. The results of PIP, PO_2 and W/D ratio

Group	PIP (mmHg)	PO_2 (mmHg)	W/D
EXP2	12.31±0.73	67.43±0.32	4.73±0.12
EXP6	16.40±0.63	57.09±0.43	5.30±0.81
EXP12	14.21±0.83	60.37±0.62	5.13±0.32
3-MA	11.20±0.35	63.35±10.20	4.83±0.82
RAPA	19.50±0.13	54.22±7.34	5.89±0.01
CON	16.31±0.81	57.17±6.33	5.28±0.73
NOR	9.40±0.84	84.23±8.34	4.34±0.67

pharmacological inhibitor and activator, respectively. The treatment with agents was carried out at the 6 h after reperfusion. The expression of Beclin-1, LC3-II were decreased in 3-MA group, but increased in RAPA group measure by western blotting compared with the control group (**Figure 5**). These results revealed that the autophagy following lung transplantation could be inhibited by 3-MA and be promoted by RAPA.

The results of TUNEL analysis showed that the apoptosis of lung cells was downregulated in 3-MA group, but was upregulated in the RAPA group compared with the control group (all $P<0.05$), which suggested that the inhibition of autophagy could decrease the apoptosis of lung cells, and that the activation of autophagy could increased the lung cell apoptosis in the rat lung transplantation model. The difference

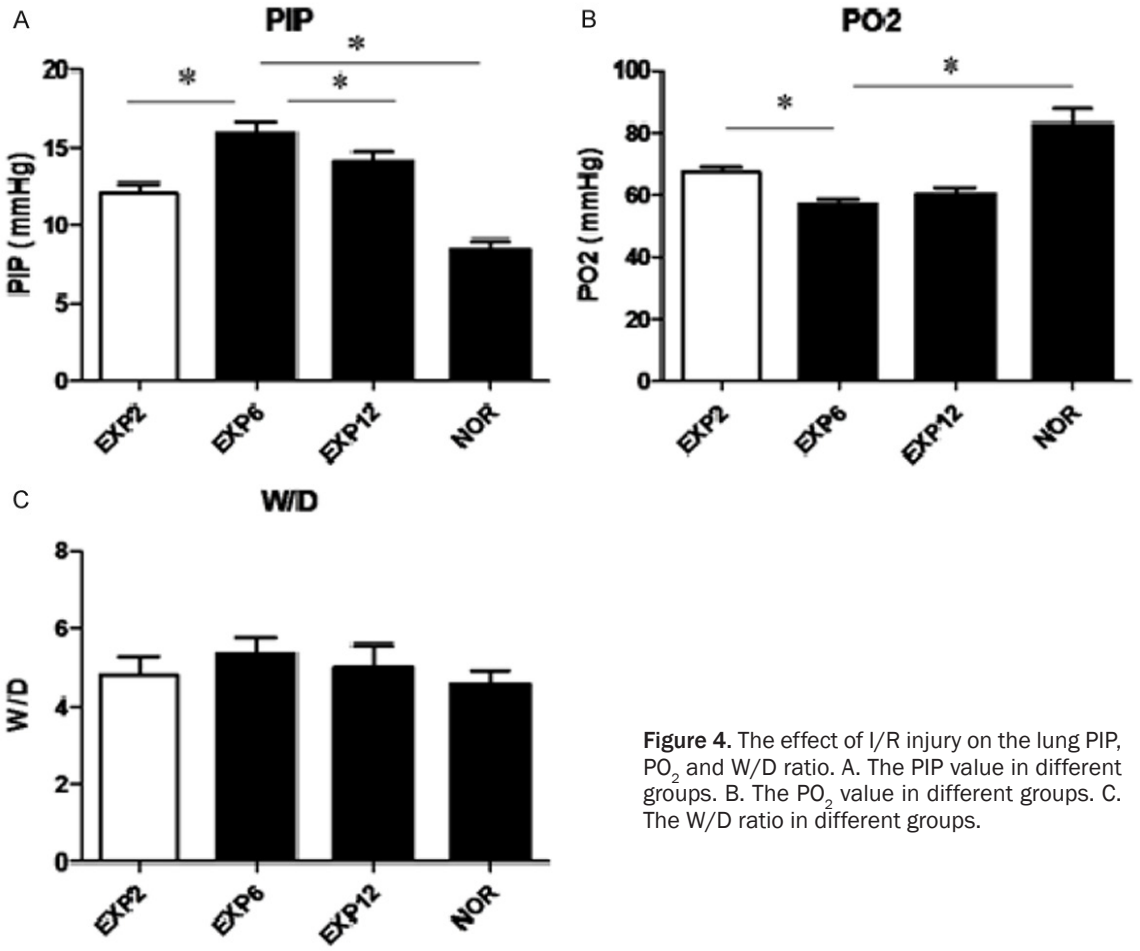

Figure 4. The effect of I/R injury on the lung PIP, PO_2 and W/D ratio. A. The PIP value in different groups. B. The PO_2 value in different groups. C. The W/D ratio in different groups.

of apoptosis between agent treatment group with control group was significantly significant (P<0.05, **Figure 6**).

After treating with 3-MA and RAPA, the PIP and W/D ratio of lung samples were significantly lower in 3-MA group than that in the control group (P<0.05), but these indices were increased in RAPA group compared to the control group (P<0.05, **Figure 7A** and **7B**). The PO_2 was upregulated in 3-MA group, while was downregulated in PAPR group compared with the control group (P<0.05, **Figure 7C**). All these results demonstrated the lung injury could be interfered by regulating the autophagy in lung samples.

Discussion

Lung transplantation represents an effective therapeutic modality for advanced lung diseases [16]. However, the survival rates following lung transplantation are not ideal due to the PGD, which is mainly caused by the I/R injury [17]. It is considered that the I/R injury usually occurs in the lung transplantation when fresh blood is reintroduced to the ischemic samples at the completion of the transplantation, leading the increased mortality of patients [18]. Thus, the molectular mechanisms of I/R injury are highlight to improve the treatments of lung disease recently years.

Autophagy involves a process of cytoplasmic proteins and acromolecules deliver from autophagosomes to lysosomes, causing the degradation in cells [19, 20]. It has been reported to involve in lots of diseases, such as cancer and so on [21, 22]. Evidences demonstrated that the autophagy can induce the non-apoptotic programmed cell death called autophagic cell death [23, 24]. The increased autophagic cell death and activity of autophagy have been detected in the damaged tissues in lots of disease models [25-28]. Despite the function of autophagy has been studied in some organs,

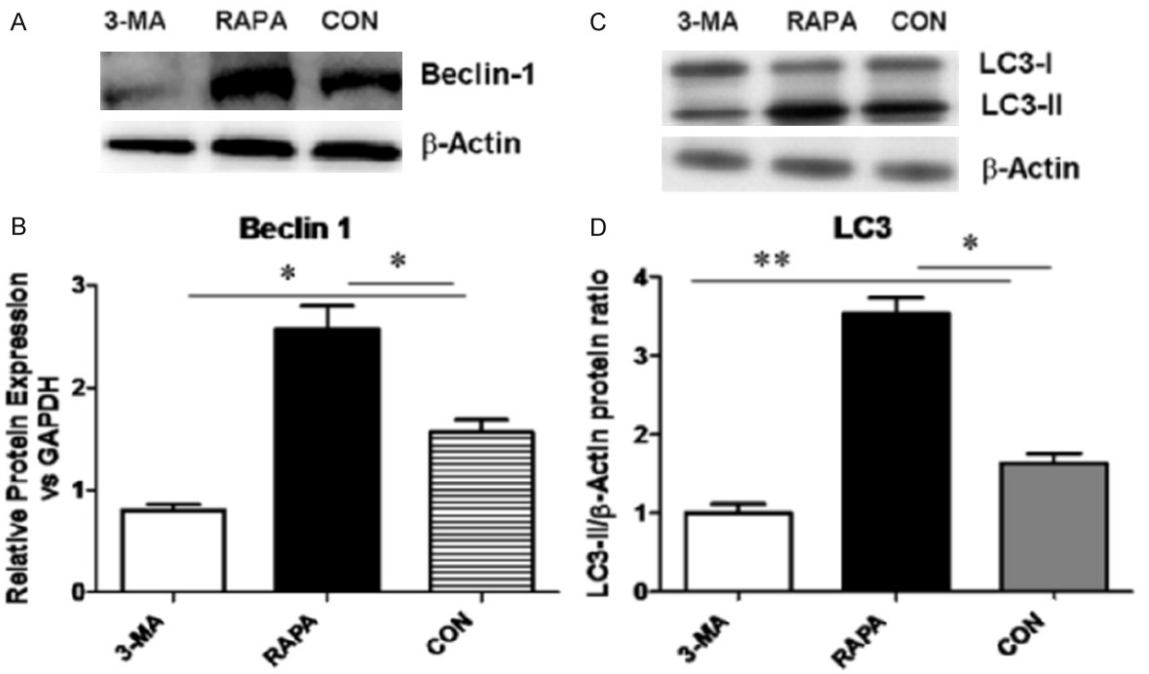

Figure 5. The expression of Beclin-1 and LC3 assessed by western blotting under the treatment with 3-MA and RAPA following lung transplantation. A. Representative immunoblots of Beclin-1 in lung. B. Beclin-1 protein levels were quantitaed by normalizing to GAPDH. C. Representative immunoblots of LC3 in lung. D. LC3-II/GAPDH ratios were quantitated by densitometry.

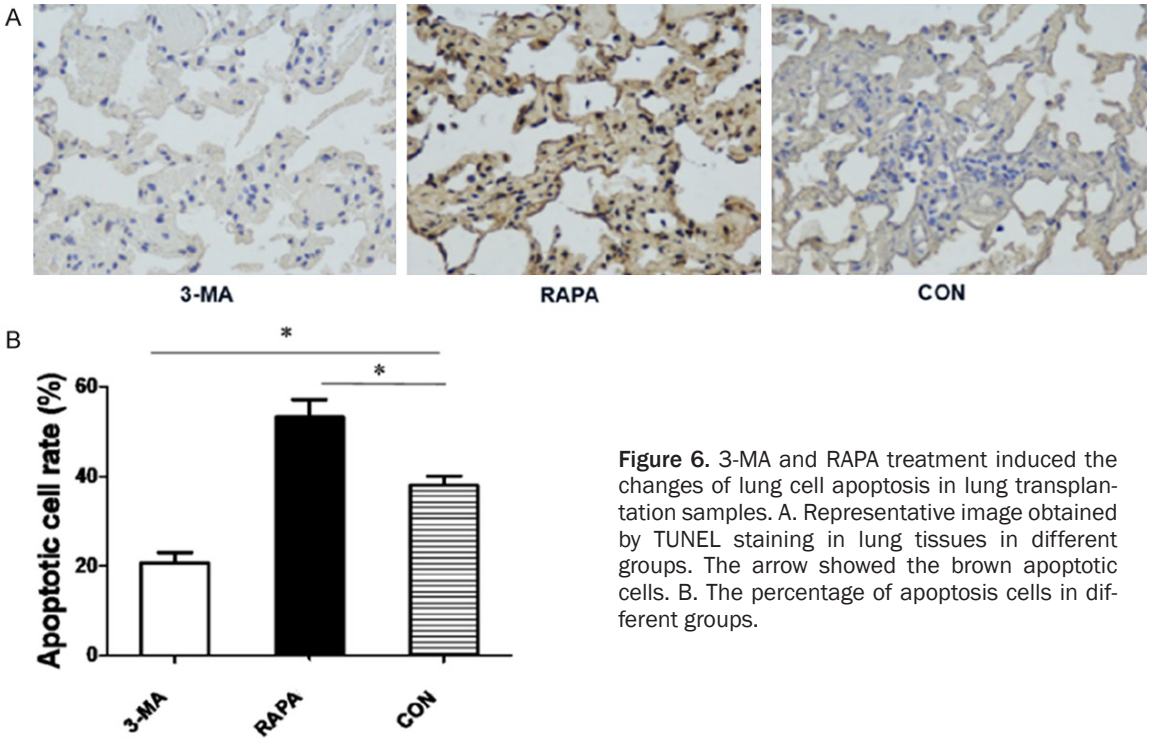

Figure 6. 3-MA and RAPA treatment induced the changes of lung cell apoptosis in lung transplantation samples. A. Representative image obtained by TUNEL staining in lung tissues in different groups. The arrow showed the brown apoptotic cells. B. The percentage of apoptosis cells in different groups.

little is known about autophagy following lung transplantation [29, 30]. The correlation of autophagy with warm I/R in lung sample has been researched in the previous study. It is

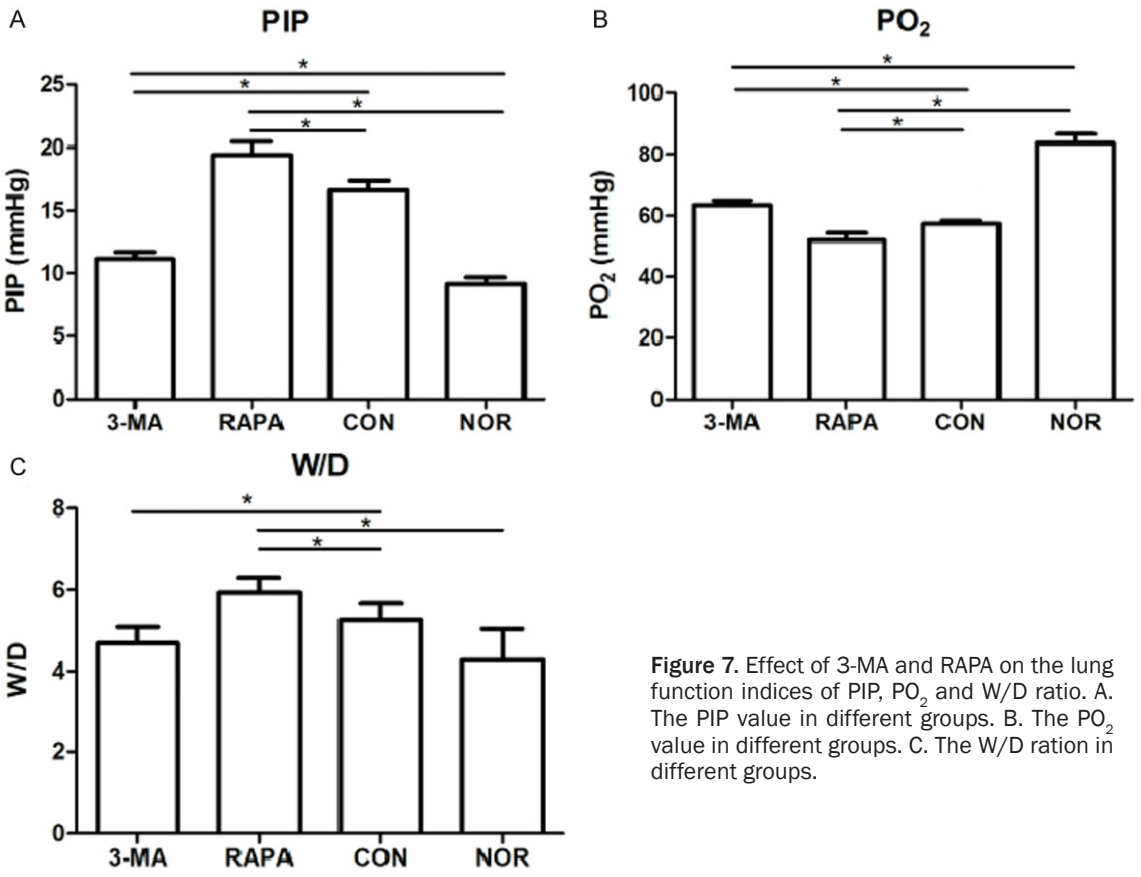

Figure 7. Effect of 3-MA and RAPA on the lung function indices of PIP, PO_2 and W/D ratio. A. The PIP value in different groups. B. The PO_2 value in different groups. C. The W/D ration in different groups.

considered that the autophagy might play a crucial role in the warm I/R in lung tissue, and the excessive autophagy can induce cell apoptosis and injury of lung function [31]. However, the I/R injury following lung transplantation is different from warm I/R, which involves the perfusion, cold ischemia and warm perfusion, named cold I/R [32]. Therefore, we aimed at to explore the role of autophagy in the cold I/R following lung transplantation. In the present study, we established the orthotopic lung transplantation model in rats to perform the function of autophagy in cold I/R injury in this lung transplantation model for improvement of lung transplantation.

In the current study, we found the autophagy was increased after lung transplantation by investigating the expression of Beclin-1 and LC3, which are the important proteins in autophagy process [33]. The results of western blotting revealed that the Beclin-1 and LC3-II were all upregulated following the lung transplantation, and peacked at the 6 h after reperfusion. As the process of autophagy is a dynamic and multi-step process, the location of autophagosomes and lysosomes by labeled Beclin-1 and LC3 can reflect the activation of autophagy and the lysosomal degration. Thus, we applied the immunofluorescence method and found the increased autophagic flux after lung transplantation, and the highest case was performed at the 6 h after reperfusion. All these data suggested that the cold I/R injury could induce the autophagy after lung transplantation. The apoptosis of lung cells were examined by using the TUNEL method. From the results, we found the increased apoptosis in the lung samples following lung transplantation. Moreover, the highest apoptosis was detected at the 6 h after lung transplantation. In order to investigate the lung injury after transplantation, we selected several indices to reveal the level of injury. The results showed that the PIP and W/D ratio were upregulated, and the PO_2 was downregulated after lung transplantation, and the peak value was found at the 6 h following transplantation.

In order to investigate whether the autophagy played crucial role in cold I/R injury in lung

transplantation model, we used 3-MA and RAPA to regulate the level of autophagy in this study. 3-MA and RAPA were the pharmacological inhibitor and activator, respectively. According the analyses, we found that the 3-MA induced downregulation of Beclin-1 and LC-II, which indicated that the inhibitor of autophagy could reduce the autophagy activity in the lung transplantation model. The activation of autophagy by RAPA increased the expression of Beclin-1 and LC-II, which suggested that the autophagy was promoted by RAPA. After the treatment with 3-MA and RAPA, the apoptosis of lung cells was assessed. Compared with the lung samples without agent treatment, the apoptosis of lung cells was decreased in 3-MA group, and was increased with the treatment of RAPA. The indices of lung function were investigated under the treatments with 3-MA and RAPA. We found that the PIP and W/D ratio were downregulated, and the PO_2 was upregulated after treatment with 3-MA compared with the non agent treatment case. Following treatment with RAPA, the PIP and W/D ratio were upregulated, and PO_2 was downregulated than that in the non agent treatment control group. Total of these results suggested that the inhibition of autophagy can suppress the cell apoptosis and lung function injury, and that the activation of autophagy can ameliorate the cell apoptosis and lung injury.

Taken together, all the data in the present study demonstrated that the authophagy was involved in the I/R injury after lung transplantation in the rats. We considered that the autophagy was activated among the process of cold ischemia and reperfusion, and could regulate the cold I/R injury following lung transplantation. The cold I/R injury could be interfered by regulating the level of autophagy in rats lung transplantation model. Although we provided the evidences of crucial role for autophagy in the cold I/R injury after lung transplantation in rats, many questions remain to assess. The mechanisms of autophagy acting in the regulation of I/R injury in lung transplantation model are needed to further investigated. We will explore the molecular biological mechanism of autophagy by study its effect on pulmonary microvascular endothelial cell (PMVEC) during lung transplantation process in the further study.

Acknowledgements

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